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I, the undersigned being an officer duly authorized in accordance with the provision of the Patent Act, 1970 hereby certify that annexed hereto is the true copy of the ***Application, Provisional & Complete Specification*** *and* ***Drawing Sheets*** *filed in connection with Application for Patent No.* ***1599/Del/2003 dated 23rd December 2003.***

Witness my hand this 7th day of February 2005.

(S.K. PANGASA)
Assistant Controller of Patents & Designs

1599 DEL 07

FORM 1

THE PATENTS ACT, 1970 23 DEC 2007
(39 of 1970)
APPLICATION FOR GRANT OF A PATENT
(See sections 5(2), 7, 54 and 135 and rule 29)

1. I/We, All India Institute of Medical Sciences, Div of Clinical Microbiology, Dept. of Laboratory Medicine, Anari Nagar, New Delhi -110 0291, India and **DEPARTMENT OF BIOTECHNOLOGY**, a Dept. of Govt. of India, CGO Complex, Lodhi Road, New Delhi 110 003

2. hereby declare -
   (a) that I am/we are in possession of an invention titled: "**METHODS FOR AMPLIFICATION AND DETECTION OF MYCOBACTERIUM TUBERCULOSIS**"
   (b) that the **Provisional Specification** relating to this invention is filed with this application.
   (c) that there is no lawful ground of objection to the grant of a patent to me/us.

3. further declare that the inventor(s) for the said invention is / are :

   (a) Singh, Sarman
   Dept. of Laboratory Medicine, Div of Clinical Microbiology, All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110 029: Indian Citizen,

   (b) Sharma, Pawan,
   Immunology Group, International Center of Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110 067: Indian Citizen

DUPLICATE

4. I/We, claim the priority from the application(s) filed in convention countries. particulars of which are as follows: Nil

5. I./We, state that the said invention is an improvement in or modification of the invention, the particulars of which are as follows and of which I/We are the applicant/patentee : **NA**

6. I/We, state that the application is divided out of my/our application, the particulars of which are given below: **Nil**
   Application No.: **Nil** and pray that this application deemed to have been filed on **NA** under section 16 of the Act.

7. That I am/We are the assignee or legal representative of the true and first inventors.

8. That my/our address for service in India is as follows:

LAKSHMIKUMARAN & SRIDHARAN.
B4/158, SAFDARJANG ENCLAVE,
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9. Following declaration was given by the inventor(s) or applicant(s) in the convention country:

I/We the true and first inventors for this invention of or the applicant(s) in the convention country declare that the applicant(s) herein is/are my/our assignee or legal representative.

Singh, Sarman	Dept. of Laboratory Medicine, Div of Clinical Microbiology, All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110 029; Indian Citizen.	
Name of first inventor	Address and Nationality	Signature
Sharma, Pawan,	Immunology Group, International Center of Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110 067; Indian Citizen	
Name of second inventor	Address and Nationality	Signature

10. That to the best of my /our knowledge, information and belief the fact and matters stated herein are correct and that there is no lawful ground of objection to the grant of patent to me/us on this application

11. Following are the attachment with the application:
(a) ~~Complete~~ Provisional specification in duplicate
(b) Drawings in duplicate
(c) Statement and undertaking on FORM-3 in duplicate
(d) Power of Attorney (To follow)
(e) Form 5
(f) CHQ for Rs 3000/- CHQ # 604407 dt 22-12-03 HDFC Bank

I/We request that a patent may be granted to me/us for the said invention.

Dated this 23 day of December, 2003

V.Lakshmikumaran
Attorney for the Applicant

To
The Controller of Patents
The Patent Office, at Delhi

FORM 2 1599 DEL 07

THE PATENTS ACT, 1970
(39 of 1970)

23 DEC 2005

PROVISIONAL SPECIFICATION
(See section 10)

"Methods for amplification and detection of

Mycobacterium tuberculosis"

ALL INDIA INSTITUTE OF MEDICAL SCIENCES, Divisional of Clinical Microbiology, Dept of Laboratory Medicine, Ansari Nagar, New Delhi -110 029, India and **DEPARTMENT OF BIOTECHNOLOGY**, a Dept. of Govt. of India, CGO Complex, Lodhi Road, New Delhi 110 003

The following specification describes the nature of the invention which it is to be performed.

Methods for amplification and detection of *Mycobacterium tuberculosis*

Field of invention

The invention provides novel oligonucleotide primers useful for amplification of ESAT-6 region for detection of *Mycobacterium* species. The primers are used for differentiating the *Mycobacterium tuberculosis* from other *Mycobacterium* species.

Background /Prior Art

According to the WHO, tuberculosis still kills 3 million individuals per year, making it the leading infectious cause of death. It is believed that one in every three individuals on the planet harbor the causative microorganism belonging to the genus *Mycobacterium* (13,24). This genus represents a complex phenotypic and genotypic diversity amongst its 84 odd species (4,9,10,14,15,17,27,28,30,34). Though, the most important human pathogenic species is *Mycobacterium tuberculosis*, other species commonly known as non- tubercular Mycobacteria or Mycobacteria other than tuberculosis (NTM or MOTT), also cause human infections in various clinical forms, adding the human suffering in terms of morbidity and mortality. The cases of HIV-TB co-infections are rapidly increasing after AIDS epidemic from both the developed and developing world (2, 10, 13, 18-21, 24). Most of these infections do not respond to the conventional anti-tubercular treatment and are considered due to infection with multi-drug resistant strains of *M. tuberculosis* (5-7, 10, 21, 26, 31, and 32). Many of these MDR labeled infections are actually not caused by the drug resistant strains of *M.tuberculsosis* but by the non- tubercular Mycobacteria.

However, in India the diagnosis of Mycobacterial infections is established empirically on clinico-radiological basis or only by sputum smear examination, and the infections caused by non-tubercular Mycobacteria are under diagnosed due to of lack of facilities. The speciation of Mycobacteria using conventional methods is very slow, labor intensive, hazardous and not always reproducible (33) hence are left un-attempted. Since AIDS cases are on steep rise in India, and HIV-TB co-infection is the commonest killer opportunistic infection in Indian AIDS patients (31), outbreaks of infections due to the NTM may not be ruled out (19). Hence it is very important to identify the causative agent to the species level for appropriate treatment.

To overcome the shortcomings of conventional methods of species specific identification of Mycobacteria, the molecular techniques are being more commonly used, in recent years. Molecular identification methods are rapid, highly sensitive and specific and can be used on a large number of samples (1, 6, 9, 16, and 33). One molecular target proved to be the most promising and commonly used is 16S rRNA gene analysis. The gene is conserved in all the species of Mycobacterial genus (1, 15 and 16). Once the genus mycobacterium is identified, the species can be differentiated by using the species specific probes, PCR primers or gene sequencing (4, 11, 22 and 28). However, identification of all the non-tubercular species of Mycobacteria may require several specific primers and repeated experimentation and most often it may not be necessary in a resource poor setting, particularly from the fresh cases, when several species can easily be identified on the basis of phenotypic characters. Therefore, Applicant's intended to separate the *Mycobacterium tuberculosis* from non-tubercular Mycobacterial group, using a rapid method. For this, a novel, simple and rapid method for differentiating MTB and NTM was developed, that uses a pair of PCR primers targeting 16S rRNA and ESAT-6 genes.

The 16S rRNA gene amplification method is a good tool to identify all the Mycobacteria. However, its further utility is restricted only for taxonomic purposes after sequencing the amplified gene product. Therefore, the single PCR method can not answer the most important clinical question, whether the Mycobacterium is *M. tuberculosis* or other than *M. tuberculosis* or the mixture of the two or many. Therefore, with clinical point of view, it is extremely important that the Clinical Microbiologist not only diagnoses a Mycobacterial infection in his patient but also provides the identification of etiological agent, whether it is M. tuberculosis or other species. On the other hand the novel PCR primers designed here target the ESAT-6 gene which is the *Mycobacterium tuberculosis* species specific coding for the early secretory antigen of 6 kDa mass (3,8, 23,25).

This antigen has been used in the humoral immunodiagnosis as well as for evaluating the cell mediated immune response against *M. tuberculosis* replacing PPD with this antigen in skin testing (3, 8). More recently, it has also been used for vaccination against tuberculosis (23,25) but its gene has never been used as target for molecular diagnosis of tuberculosis.

The antigen- antibody based test methods in which a specific antibody against a known antigen (ESAT-6 antigen in this case) has a fundamental drawback of poor predictive value to make an organ specific disease diagnosis. The detection of antibodies against ESAT-6, though have been found very specific in tuberculosis non-endemic countries, its utility in TB endemic countries such as whole of Asia, Africa, Russia and other middle Asian countries is very limited due to sub clinical exposures of the population. Obviously all the exposed persons will have circulating antibodies in their blood against this antigen. Therefore, if a pre-exposed symptomatic person gets fresh brain infection with TB and the *Mycobacterium tuberculosis* colonises and causes brain tuberculoma, the antibody detection assays will be of no use in such cases. To explain it in other words, antibody detection assays, for diseases of high endemicity (eg. Tuberculosi which is airborne) have very poor specificity and organ specific diagnosis can be made at all. Since, the antibody detection methods are indirect evidences of infection.

On the other hand the molecular methods such as PCR are highly specific because in these methods the genome of the living organism from the specific diseased site is detected. In other words, using PCR, the specific diagnosis of tubercular meningitis, abdominal tuberculosis, gastrointestinal tuberculosis, genitourinary tuberculosis beside the pulmonary tuberculosis can be made. Moreover the biggest advantage of molecular methods is that the DNA of causative agent (Mycobacterium) can be detected from old samples such as mummies, fossils etc. while the antibodies can not.

The present work shows its utility in species-specific and rapid molecular diagnosis of tuberculosis using *esat*-6 gene amplification, for the first time.

Brief description of accompanying drawing

Figure 1 A:

The PCR was carried out as per the detailed protocol mention in the material and method for genus specific molecular identification of the growth, targeting 16s rRNA gene. The PCR product of 1030 bp size was resolved on 0.9% agarose gel. Lanes 1, 11, & 22 are 100 bp molecular weight markers. Lanes 2, 12, & 23 are H37rv standard strain of *Mycobacterium tuberculosis*. Lane 3-10 are standard non-tubercular Mycobacteria (as shown in table 1), 13-21 & 24-26 are clinical isolates.

Figure 1 B:

The isolates which were identified as Mycobacteria on the basis of genus specific 16srRNA PCR and conventional identification methods were subjected to *Mycobacterium tuberculosis* species specific ESAT-6 PCR (see material and methods). The PCR product of 320 bp size was resolved on 1.5% agarose gel.

Lanes 1, & 17 are 100 bp molecular weight markers. Lanes 2, & 18 are H37rv standard strain of *Mycobacterium tuberculosis*. Lane 3-15, & 19-24 are the same clinical isolates as shown in panel A. The lane 16 is *M.bovis* showing no amplification. Remaining 7 standard strains of non-tubercular Mycobacteria (as shown in table 1) also did not show any amplification thus not shown here.

Description of the invention

Of the 265 patients included in this study, only 85 (32.0%) grew Mycobacteria in the L-J culture. Their clinical and laboratory data were analyzed to ascertain the sensitivity of various diagnostic methods (Table 1). Other patients whose cultures remained negative after 10 weeks of incubation were excluded from this study. These 85 patients could be divided into four groups on the basis of clinical disease manifestations and their HIV status. Seventy patients were HIV negative and 15 were HIV-1 positive. Out of the 70 HIV negative patients, 24 patients presented with lymphadenitis, and remaining 46 had pulmonary manifestation. Of these only 22 were new cases who did not receive any anti-tubercular treatment while other 24 were bacteriologically confirmed cases of pulmonary tuberculosis and were on anti-tubercular treatment but without response. Because, these patients continued to excrete the acid fast bacilli in their sputum even after 6 months of treatment with Isoniazide, Rifampicine, Ethambutol and Pyrazinamide, these cases were labeled as MDR patients. The mean month time for anti-tubercular treatment in these patients was 26.7 ± 17, the maximum being 6 years. All HIV infected patients included in this study presented with pulmonary manifestations.

Overall the detection rate of L-J culture in our laboratory was 32% (85/265). Similarly, the detection rate of Ziehl-Neelson staining was only 19.6%, and that of Auramine-O staining 22.3%. However, the sensitivity of A-O staining and ZN stained smears on

culture proven cases was 75% and 66.6% for MDR cases, 72.7% and 68.2% for new pulmonary tuberculosis cases, 66.6% and 58.3% for tubercular lymphadenitis and poor 60% and 46.6% in HIV infected pulmonary tuberculosis cases, respectively. Hence the AO stain had clear edge over Z-N stain and culture had highest detection rate.

On culture examination, 86 strains could be isolated from the 85 samples. One sample grew two types of colonies one being rapid grower. On PCR examination, all 86 cultures were found positive by genus specific conserved gene, 16S rRNA. The 16S rRNA PCR products were resolved on a 0.9% agarose gel after ethidium bromide staining. The amplicons of 1030 bp size indicated genus specific positive result (Figure 1, panel A). However, out of 86 (85 plus two types of growth in one patient) colonies only 67 (77.9%) isolates showed 320 bp amplification of the *Mycobacterium tuberculosis* species specific ESAT-6 gene. The patient, from whom two types of Mycobacterial colonies grew, was on anti-tubercular treatment for the past 4 years but without any significant clinical benefit. Both the colonies were subjected to colony characterization following conventional methods as well as the polymerase chain reaction and 16S rRNA-sequencing (29). One of the two colonies did not contain the ESAT-6 gene, though genus specific PCR was positive. The non-tubercular colony, which was ESAT-6 PCR negative, was identified as *M. smegmatis while* the other as *M. tuberculosis* (29).

On the basis of genus and species specific primers used in this study, the Applicants found very high rate of non-tubercular Mycobacteria in HIV infected patients (33.3%, 5/15). It was closely followed by patients with lymphedenitis (29.1%, 7/24) and patients unresponsive to antitubercular treatment (20.8%, 5/24). These findings were highly significant ($p < 0.001$) while comparing the incidence of non-tubercular Mycobacteria in all the above groups with prevalence in new cases (4.5%). All the 18 non-tubercular Mycobacteria have been identified phenotypically and genotypically.

The All India Institute of Medical Sciences (AIIMS), New Delhi is a tertiary care hospital and most of our patients are already treated for tuberculosis empirically at local peripheral level. As a result less number of cases visit this hospital directly without taking prior treatment. As our figures show that more than half (52.2%) of these patients were taking treatment but without improvement. The prevalence of non-

tubercular Mycobacteria in these patients was significantly ($p < 0.001$) higher than new cases (20.8% vs 4.5%). This finding is in concurrence with another major study published recently from south India (21). In this study, the prevalence of MDR in newly diagnosed cases of pulmonary tuberculosis was 2.5% while in previously treated cases it was 81.2% of the *M.tuberculosis* isolates (21). Our study also indicates that 79.2% cases were MDR-*M.tuberculosis* while the rest 20.8% cases did not respond because the infection was caused by non-tubercular Mycobacteria.

As expected, in the present study, the prevalence of non-tubercular Mycobacteria was very high in AIDS associated pulmonary tuberculosis. In another study (5) carried out from south India on HIV-TB co-infection, recently, showed that in AIDS associated tuberculosis the primary unresponsiveness to anti-tubercular treatment was as high as 33.9%. We also found that our 33.3% patients had non-tubercular Mycobacteria, most of them identified to be *Mycobacterium avium-intracellulare* using 16s rRNA sequencing (29). Since the Non-tubercular Mycobacteria are not susceptible to conventional anti-tubercular treatment, it is always desirable to identify these isolates up to species level, particularly from drug resistant cases. Though, the earlier study from south India (5) did not specify the causative species from unresponsive AIDS patients, our findings are another milestone in this direction, clearly emphasizing the need for speciation of the causative species of Mycobacteria. The present study countermands the general belief that all non-responding cases in India are due to MDR-TB. Our data indicates that more than one quarter of these patients who do not respond to primary ATT are infected with non-tubercular Mycobacteria.

M. tuberculosis has been reported a leading cause of lymphadenitis in developing world and the USA (26, 33). The data from India is scarce and only a few case reports are available and the speciation is based only on phenotypic features which have several limitations. The high rate (29.2%) of non-tubercular Mycobacteria causing lymphadenitis in present study could, therefore, be explained on the basis of highly sensitive and *M. tuberculosis* specific molecular method used here. Also most of the non-tubercular Mycobacterial species were identified as *Mycobacterium avium* complex (details not shown here and will be published elsewhere). Further, using 16s rRNA sequencing, all the non-tubercular species could be speculated.

Out of the total 100 patients suspected to have HIV-TB co-infection and included in this study for investigations, only from 15 patients Mycobacteria could be isolated. Other workers have also reported low culture positivity in HIV infected persons (20, 22). The high frequency of non-tubercular Mycobacteria in AIDS patients is well documented from developing countries (2, 10, 18, and 19) but not much work is published from India. This may be due to lack of facilities to identify the species of Mycobacterial isolates. Therefore, the present study is a documentation of high infection rate of AIDS patients with non-tubercular Mycobacteria diagnosed using a rapid, non-hazardous and sensitive PCR method. In another study the applicants used the same set of PCR primers, directly on the clinical samples. The Mycobacterial DNA detection rate in that study was 100% on all the sputum samples taken from confirmed cases of pulmonary tuberculosis and no false positive.

Experimental Procedures

Clinical Samples:

Clinical samples were obtained from patients attending the out patient department (OPD), or hospitalized patients at the All India Institute of Medical Sciences (AIIMS), New Delhi, India. All the samples were processed in the Clinical Microbiology Division, Department of Laboratory Medicine. A total of 3072 patients who attended the general OPD of AIIMS between April 1999 and March 2001, with complaints of fever, cough and expectoration were investigated to rule out tuberculosis, as a routine work-up. However, only 265 of these patients had clinico-radiological findings suggestive of tubercular etiology and were referred to specialized clinics. These referred patients fulfilled the inclusion criteria as per CDC guidelines and were included in this study. After informed consent of the patients, their routine investigations were carried out and their blood samples were also tested for human Immunodeficiency virus (HIV) infection. The clinical samples investigated for Mycobacterial isolation included sputum, lymph node aspirate or biopsy specimens and pleural fluid, according to the clinical manifestations. In case of sputum, 1-3 samples from each patient were obtained, while for other specimens sampling was done only once. However, repeat samples from the same patient were not counted as different, even if one or all the three sample from one patient were culture positive, it was counted as one only. All samples were processed on the same day of receipt. The sputum samples were decontaminated and concentrated using Petroff's method (12) within 24 hours of receipt. The

decontaminated samples were inoculated on L-J culture slants. One aliquot of the pellet was stored at -20°C until further use. From the remaining pellet slide smears were prepared.

Microscopy and routine culture:

The slide smears were air dried and heat fixed. One set of smears was stained with auramine-O (A-O) fluorochrome staining and examined under epifluorescence at 400X using Nikon®™ fluorescent microscope and the another set stained with Zeihl-Neelsen staining and examined under oil emersion (1000X). The results and quantitatation of acid fast bacilli (AFB) were reported as per revised WHO guidelines (33): scanty (1 or 2 AFB in 300 oil fields), 1+ (3-9 AFB in 100 fields), 2+ (1-10 AFB per oil field), or 3+ (10 or more AFB per oil field).

Culture and identifications were made according to the standard method (12, 36) by inoculating on a Lowenstein-Jensen slant with 0.2 to 0.5 ml of the decontaminated sputum sample. Cultures were incubated at 37°C till the growth or for 8 weeks whatsoever was early. The cultured Mycobacteria were identified by staining and standard biochemical methods including Niacin, heat stable catalase, aryl sulphatase and Tween-80 hydrolysis tests. The PCR analysis was done using the 16S rRNA primers and ESAT-6 primer sets.

Extraction of genomic DNA for PCR:

The DNA was isolated directly from the Mycobacterial growth by a procedure which the applicants have been doing in our laboratory for the last several years. Briefly: 2-3 loopful (~100mg) of Mycobacterial growth was transferred to an eppendorf tube containing 200 µl of sterile distilled water. The suspension was incubated at 80°C in a water bath for 20 minutes. To the suspension 200 µl chloroform was added followed by vortexing. The suspension was again incubated at 65 °C for 10 minutes and centrifuged at 9000 rpm for 2 minutes. The clear supernatant containing Mycobacterial DNA was taken for PCR.

Genus specific PCR Primers:

Primers for the amplification of a 1030bp 16S rRNA gene were taken, as described elsewhere (14, 15, 28, and 33). The primers for16S rRNA were

Primer 16SrRNA 285: 5' gag agt ttg atc ctg gct cag 3' and
Primer 16S rRNA 264: 5' tgc aca aca ggc cac aag gga 3'.

M. tuberculosis specific PCR

All the samples were also subjected to *M. tuberculosis* specific PCR, using primers designed to amplify full length *esat-6* (Rv3875) gene. These novel primers have been designed for the first time for species specific PCR based diagnosis of *Mycobacterium tuberculosis*. The primers also contained restriction sites for cloning in an expression vector in a related study.

The primers were:

ESAT-6F: 5' gcg gat ccc atg aca gag cag cag tgg a 3' (BamHI site underlined)

and

ESAT-6R ccc aag ctt cct atg cga aca tcc cag tga cg 3' (HinDIII site underlined)

The PCR fragment obtained with these primers from genomic DNA of a local clinical isolate of *M.tuberculosis* was cloned into an intermediate vector pGEM-T easy® (Promega) and its nucleotide sequence ascertained. The nucleotide sequence obtained was identical to that published by Cole *et al* (4). The sequence has been deposited with the GenBank with accession no. AF420491.

PCR:

Mycobacterial DNA (50ng) was amplified in a 50 µl reaction mixture for the amplification of 16S rRNA gene The reaction mixture contained, 200µM deoxynucleoside triphosphates, (Bangalore Genei, Pvt. Ltd., Bangalore, India), 5µl of 10X buffer [100mM TAPS (pH 8.8), 15mM $MgCl_2$, 500mM KCl and 0.1% gelatin] and 1.5 units of *Taq* DNA polymerase. The working concentration of each primer was 0.5µM. The temperature cycles used were: 94^0C for 5 minutes; 94^0C for 1 minute, 62^0C for 2 minutes and 72^0C for 2 minutes. A total 40 amplification cycles were carried out followed by final extension at 72^0C for 10 minutes. The PCR products were resolved on a 0.9% agarose gel after ethidium bromide staining. The amplicons of 1030 bp size indicated positive result (Figure 1, panel A).

The constituents for species-specific ESAT-6 PCR mixtures were same as for the genus specific 16S rRNA PCR, except that the reaction was carried out for 30 cycles of 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C; followed by final extension at 72°C for 10

min. The products were electrophoreses in 1.5% agarose gels, to resolve the PCR product of 320bp (Figure 1, Panel B)

For control, other standard species of Mycobacterium were also studies. Following non-tubercular mycobacterial species were included:

Table 1.

Mycobacterial species	16SrRNA PCR result	esat-6 PCR result
M. tuberculosis	+	+
M. bovis	+	-
M. avium	+	-
M. smegmatis	+	-
M. duvalli	+	-
M. fortuitum	+	-
M. celatum	+	-
M. austroafricanum	+	-
M. flavescence	+	-

The above table clearly shows that the *esat-6* primers were highly specific for *M. tuberculosis*. Even though primers specific for Mycobacterium tuberculosis complex (which includes *M. bovis*) have been reported ours is the first invention which can rule out even the *M .bovis* post BCG vaccination infection. In India BCG (Bacillus of Calmette and Guerin) vaccineation is given in early childhood to all children. The vaccine contains mutated BCG strain of *Mycobacterium bovis*. So far there are no rapid and direct molecular methods of making exclusive diagnosis of *M. tuberculosis* infection minus *M. bovis*. Moreover, the limitation of antibody detection assays using ESAT-6 antigen is enormous in India. The *Mycobacterium bovis* is also an important cause of tuberculosis is animals (cattles) of India and traditionally the Indian farmers and who consume raw cow milk will have antibodies against this bacteria. Therefore the antibody detection methods will give false positive report of tuberculosis in otherwise healthy individuals and many patients may undergo unnecessary anti-tuberculosis treatment while not treating the actual disease. Therefore, our invention will revolutionise the M. tuberculosis species specific diagnosis by using multiplex PCR (using 16SrRNA and *esat-6* primers) directly on the clinical samples or using single *esat-6* primers on the culture isolates.

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Table 2. Detection rate of Mycobacterial etiology in 4 patient groups using various conventional and molecular methods.

Patient Group	Culture isolates	Biochemical identification		PCR identification			
				16s rRNA		Esat-6	
		M.tb	NTM	+	-	+	-
AIDS	15	10 (66.6%)	5 (33.3%)	15	0	8	7
Lymphadenitis	24	19 (79.2%)	5 (20.8%)	24	0	17	7
MDR-TB	24+1*	19 (76.0%)	6 (24.0%)	25	0	19	6
Rx Naïve	22	21 (95.5%)	1 (4.5%)	22	0	21	1

Total	85+1* 20	69 (81.2%)	16 (18.8%)	85	0	65

* from one patient, two types of isolates were obtained. One was identified as *M. tuberculosis* and the other as *M.smegmatis*.

AIDS, Aquired Immunodeficiency Syndrome; M.tb, *Mycobacterium tuberculosis;* NTM, Non-tubercular Mycobacteria; MDR-TB, Multidrug resistant tuberculosis.

The Non-tubercular Mycobacteria were identified by gene sequencing and the sequences have been submitted to GenBank with Accession no AF419854, AF504932, AF504931, AF504926, AF498754, AF498317.

Dated 23rd day of December 2003

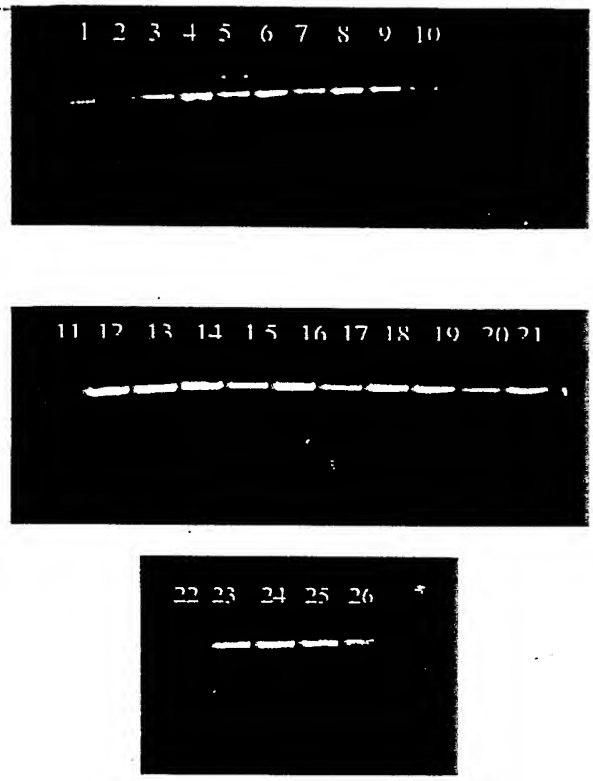
V.LAKSHMI KUMARAN
Attorney for the Applicants

To

THE CONTROLLER OF PATENTS,
Patent Office,
Delhi

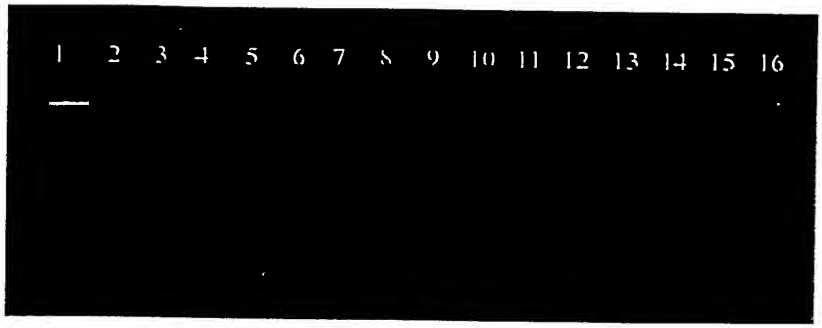
Representative agarose gel electrophoresis of the PCR products

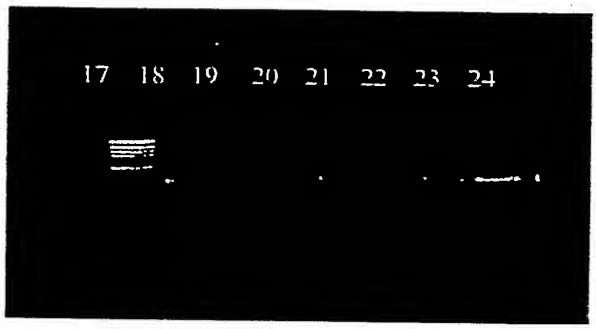
Fig 1 A



Representative agarose gel electrophoresis of the PCR products

Fig 1 B





FORM 2

THE PATENTS ACT, 1970
(39 of 1970)

COMPLETE SPECIFICATION
(See section 10)

"Methods for detection of *Mycobacterium tuberculosis*"

ALL INDIA INSTITUTE OF MEDICAL SCIENCES, Divisional of Clinical Microbiology, Dept of Laboratory Medicine, Ansari Nagar, New Delhi -110 029, India; and **DEPARTMENT OF BIOTECHNOLOGY,** a Dept. of Govt. of India, CGO Complex, Lodhi Road, New Delhi 110 003

The following specification particularly describes the nature of this invention and the manner in which it is to be performed

Methods for detection of *Mycobacterium tuberculosis*

TECHNICAL FIELD

The invention provides novel oligonucleotide primers for the amplification of Early Secretory Antigenic Target (ESAT)-6 regions for detection of *Mycobacterium* species. The primers are used for differentiating the *Mycobacterium tuberculosis* from other species of *Mycobacterium*. Further, the invention provides a method for detection of *Mycobacterium tuberculosis* based on the DNA amplification of the ESAT-6 region.

BACKGROUND AND PRIOR ART

According to the WHO, tuberculosis still kills 3 million individuals per year, making it the leading infectious cause of death. It is believed that one in every three individuals on the planet harbor the causative microorganism belonging to the genus *Mycobacterium* (13, 24). This genus represents a complex phenotypic and genotypic diversity amongst its more than 100 odd species (4,9,10,14,15,17,27,28,30,34). Though, the most important human pathogenic species is *Mycobacterium tuberculosis* (M.tb), other species commonly known as non- tubercular Mycobacteria (NTM) or Mycobacteria other than tuberculosis (MOTT) also cause human infections in various clinical forms, particularly in immunosuppressed patients, adding to the human suffering in terms of morbidity and mortality. The cases of HIV-TB co-infections are rapidly increasing, after the AIDS epidemic, from both the developed and developing world (2, 10, 13, 18-21, 24). Most of these non-Tubercular Mycobacteria are not susceptible to the conventional anti-tubercular treatment and are wrongly considered due to infection with multi-drug resistant (MDR) strains of *M. tuberculosis* (5-7, 10, 21, 26, 31, 32). Many of these MDR labeled infections are actually not caused by the drug resistant strains of *M.tuberculsosis* but by the non- tubercular Mycobacteria. HIV-Mycobacteria co-infection is the most common killer opportunistic infection in Indian AIDS patients (31), but the outbreaks of infections due to the NTM in these severely immunocompromised population, can not be ruled out (19). Hence it is very important to identify the causative agent to the species level for appropriate treatment.

However, in India the diagnosis of Mycobacterial infections is established empirically on clinico-radiological basis or only by sputum smear examination, and the infections caused by non-tubercular Mycobacteria are under-diagnosed due to lack of diagnostic facilities. The speciation of *Mycobacteria* using conventional methods is very slow, labor intensive, hazardous and not always reproducible (33) and hence left un-attempted by most of the Indian Laboratories.

To overcome the shortcomings of conventional methods of species specific identification of *Mycobacteria*, the molecular techniques are being more commonly used, in the recent years. Molecular identification methods are rapid, highly sensitive and specific and can be used on a large number of samples (1, 6, 9, 16, 33, US Patent No. 5652106, US Patent No.5731150). One molecular target which has proved to be the most promising and commonly used is the 16S rRNA gene analysis. This gene is conserved in all the species of Mycobacterial genus (1, 15, 16, US Patent No. 5811269). Once the genus mycobacterium is identified, the species can be differentiated by southern hybridization using the species specific probes, or gene sequencing (4, 11, 22, 28). However, identification of all the non-tubercular species of *Mycobacteria* may require several specific primers and repeated experimentation and most often it may not be necessary in a resource poor setting, particularly from the fresh cases, when several species can easily be identified on the basis of phenotypic characters. Therefore, the present invention intends to differentiate the *Mycobacterium tuberculosis* from non-tubercular Mycobacterium species, using a rapid PCR amplification method. For this, a novel, simple and rapid method for differentiating MTB and NTM has been developed, that uses a pair of oligonucleotide primers targeting the *esat*-6 and 16S rRNA genes.

The 16S rRNA gene amplification method is a good tool to identify all the *Mycobacteria*. However, its further utility is restricted only for taxonomic purposes after sequencing the amplified gene product. Therefore, the single PCR method can not answer the most important clinical question, whether the Mycobacterium is *M. tuberculosis* or other than *M. tuberculosis*. Therefore, with clinical point of view, it is extremely important that the Clinical Microbiologist not only diagnoses a Mycobacterial infection in his patient but also provides the identification of the etiological agent, whether it is *M. tuberculosis* or other species of *Mycobacterium* for which the treatment protocol is totally different from the first. On the other hand, the novel oligonucleotide primer pair designed in the present invention target the *esat*-6 gene which is *Mycobacterium tuberculosis* specific gene coding for the early antigen of 6 kDa mass (3,8, 23,25). Though this antigen has been used in the humoral immunodiagnosis as well as for evaluating the cell mediated immune response against *M. tuberculosis*, replacing PPD with this antigen in skin testing (3, 8). More recently, it has also been used for vaccination against tuberculosis (23, 25, US Patent No.6649170) but its gene has never been used as a target for molecular diagnosis of tuberculosis. This gene is deleted in species of Mycobacteria other than tuberculosis complex (4).

The antigen- antibody based test methods have been a research topic but no antigen has been found to be satisfactory. Most of the genus-specific antigens failed because they cross reacted with the environmental *Mycobacteria* and with BCG which is given as a vaccine. Even the *M. tuberculosis* specific antigen (ESAT-6 antigen in this case) has a fundamental drawback of poor predictive value to make an organ specific disease diagnosis. The detection of antibodies against ESAT-6 protein has been found to be somewhat useful in tuberculosis non-endemic countries, its utility in TB endemic countries such as whole of Asia, Africa, Russia and other South American countries is very limited due to sub-clinical exposures of the population . Obviously all the exposed persons will have circulating antibodies in their blood against this antigen. Therefore, if a pre-exposed asymptomatic person (antibodies already positive) gets fresh TB brain infection (TB meningitis) and in another pre-exposed person there is no such fresh infection, the antibody detection assays will not be useful for the specific diagnostic use in such cases, as both these patients will be positive for these antibodies. To explain it in other words, antibody detection assays, for diseases of high endemicity (e.g. Tuberculosis which is airborne) have very poor specificity and organ specific diagnosis can not be made at all, as the antibody detection methods are indirect evidences of infection.

On the other hand the molecular methods such as PCR are highly specific because in these methods the genome of the living organism from the specific diseased site is detected. In other words, using PCR amplification, the specific diagnosis of tubercular meningitis, abdominal tuberculosis, gastrointestinal tuberculosis, genitourinary tuberculosis besides the pulmonary tuberculosis can be made. Also the PCR amplification will detect only active diseases and not the old exposures. Moreover the biggest advantage of molecular methods is that the DNA of causative agent (Mycobacterium) can be detected from old samples such as mummies, fossils etc. while the antibodies can not.

The present invention shows its utility in species-specific and rapid molecular diagnosis of tuberculosis using *esat*-6 gene amplification, for the first time.

Definitions of certain terms used in the specification

AIDS - Acquired Immunodeficiency Syndrome;

M.tb- *Mycobacterium tuberculosis;*

NTM- Non-tubercular Mycobacteria;

MDR-TB- Multi drug resistant tuberculosis.

BRIEF DESCRIPTION OF ACCOMPANYING DRAWING

Figure 1 The PCR method for genus-specific identification of the *Mycobacterium* species based on the 16s rRNA gene. Lanes 1, 11, & 22 are 100 bp molecular weight markers. Lanes 2, 12, & 23 are H37rv standard strain of *Mycobacterium tuberculosis*. Lanes 3-10 are standard non-tubercular *Mycobacteria* (as shown in table 1), 13-21 and 24-26 are clinical isolates.

Figure 2: The isolates which were identified as *Mycobacteria* on the basis of genus-specific 16s rRNA PCR were subjected to amplification based on the ESAT-6 region. Lane 1 & 17 is 100 bp molecular weight markers. Lane 2 & 18 is H37rv standard strain of *Mycobacterium tuberculosis*. Lane 3-15 & 19-24 is the same clinical isolates as shown in panel A. The lane 16 is *M. bovis* showing no amplification.

OBJECT OF THE INVENTION

The main object of the present invention is to develop an oligonucleotide primer pair for specific amplification of the Early Secretory Antigen of Target *(esat)-6 gene* of *Mycobacterium* species

Another object of the present invention is to develop a method for detecting M. tuberculosis in a sample based on the amplification of *esat*-6 gene using the primer pair.

Still another object of the present invention is to develop a method for detecting M. tuberculosis wherein the amplification is done by polymerase chain reaction.

Further object of the present invention is to develop a diagnostic kit for detecting *M. tuberculosis*, based on the amplification of *(esat)*-6 gene.

SUMMARY OF THE INVENTION

The present invention provides novel oligonucleotide primer pair having SEQ ID NO: 3 and SEQ ID NO: 4 for amplification of Early Secretory Antigenic Target (*esat*)-6-gene of *Mycobacterium* species. The invention also provides a method for detecting *M. tuberculosis* in a sample based on the amplification of *esat*-6 gene, comprising isolating DNA template from the sample, amplifying with the above oligonucleotide primer pair and subjecting the amplified DNA product to separation and staining to detect the presence of amplified DNA product for identifying *Mycobacterium tuberculosis* in the sample. The invention further provides a diagnostic kit for detection of *Mycobacterium tuberculosis*. The invention also provides a method of detecting *Mycobacterium tuberculosis* from a sample by amplifying the 16s rRNA region from the isolated DNA template by conventional

methods to detect *Mycobacterium* species and further amplifying the positive sample containing *Mycobacterium* species using novel oligonucleotide primer pair for amplification of *ESAT*-6 region of a band of 320 bp which is indicative of the presence of *Mycobacterium tuberculosis*.

DETAILED DESCRIPTION OF THE INVENTION

In accordance, the present invention provides for an oligonucleotide primer pair for amplification of the Early Secretory Antigen of Target (*esat*)-6 gene comprising of SEQ ID NO: 3 and SEQ ID NO: 4.

In an embodiment, the present invention provides a method for detecting *M. tuberculosis* using the oligonucleotide primer pair, the said method comprising the steps of:

a) isolating DNA template from the sample,

b) amplifying the DNA template by adding a reaction buffer, oligonucleotide primer pair having SEQ ID NO: 3 and SEQ ID NO: 4, and heat stable DNA polymerase to obtain an amplified DNA product, and

c) subjecting the amplified DNA product of step (b) to separation, and staining to detect the presence of amplified DNA product wherein the presence of amplified DNA product is indicative of *Mycobacterium tuberculosis* in the sample.

Further embodiment of the present invention is for a method for detecting *M. tuberculosis*, based on the amplification of the reaction mixture containing DNA from sample, oligonucleotide primer pair having SEQ ID NO: 3 and SEQ ID NO: 4, reaction buffer, dNTPs and DNA polymerase preferably *Taq* polymerase.

Another embodiment of the present invention is for a method for the detection of *M. tuberculosis* from either a clinical or a culture sample, wherein the clinical samples are selected from sputum, bronchoalveolar lavage fluid, pleural fluid, ascetic/peritoneal fluid, cerebrospinal fluid (CSF), pus, faecal matter, urine, amniotic fluid, menstrual blood, peripheral blood or other body fluids, lymphnode, pus or other aspirate and tissue biopsies.

Further embodiment of the present invention is for a method for detecting *M. tuberculosis* using the oligonucleotide primers having SEQ ID NO: 3 and SEQ ID NO: 4, the DNA sample obtained from Mycobacterial cultures

Yet another embodiment of the present invention is for a method for detecting *M. tuberculosis* wherein the amplification is done by polymerase chain reaction.

Still another embodiment of the present invention is the presence of the amplified DNA product is of 320 bp in size which is indicative of the *M. tuberculosis*.

In yet another embodiment of the present invention is for a diagnostic kit for detection of *M. tuberculosis*, based on the amplification of *(esat)-6* gene. The kit further comprises of oligonucleotides primer pair having SEQ ID NO; 3 and SEQ ID NO: 4, reaction buffer, DNA polymerase preferably *Taq* polymerase, negative and positive control, DNA marker, deoxyribonucleictriphosphates (dNTPs) and an instruction manual.

A further embodiment of the present invention is for a method for detecting *M.tuberculosis* based on amplification wherein, the said method comprising the steps of:

i. amplifying the 16s rRNA region from the isolated DNA template using the primer pair having SEQ ID NO: 1 and SEQ ID NO: 2 to obtain first amplified product using conventional method,

ii. detecting the amplified product of step (a) wherein the presence of 1030 base pair amplified DNA product is indicative of positive sample for the presence of *Mycobacterium* species,

iii. employing the DNA from the positive samples identified from step (b) for further detection of *M. tuberculosis* based on the amplification of *esat*-6 gene,

iv. amplifying the *esat*-6 gene using the primer pair having SEQ ID NO: 3 and SEQ ID NO: 4 to obtain second amplified product using method as claimed in claim 2,

v. detecting the amplified product of step (d) wherein the presence of 320 base pair is indicative of *Mycobacterium tuberculosis* in the sample and absence is indicative of *Mycobacterium* species other than *M.tuberculosis*.

The present invention teaches a method for detection of *M.tuberculosis* based on amplification of *esat*-6 gene. This invention provide the novel oligonucleotide primer pair & the method of detection which is given below:.Two hundred and sixty five patients were taken up for this study for detection of Mycobacterial infection. Their clinical and laboratory data were analyzed to ascertain the sensitivity of various diagnostic methods (Table 1). Only 85 patients (32.0%) showed the presence of *Mycobacteria* in their L-J culture (See Example 1 for culturing of *Mycobacterium*). Other patients whose cultures remained negative after 10 weeks of incubation were excluded from this study. These 85

patients could be divided into four groups on the basis of clinical disease manifestations and their HIV status. 70 patients were HIV negative and 15 were HIV-1 positive. Out of the 70 HIV negative patients, 24 patients were present with lymphadenitis, and the remaining 46 had pulmonary manifestation. Of these only 22 were new cases who had not received any anti-tubercular treatment while the other 24 were bacteriologically confirmed cases of pulmonary tuberculosis and were on anti-tubercular treatment but without response. Because, these patients continued to excrete the acid fast bacilli in their sputum even after 6 months of treatment with Isoniazide, Rifampicin, Ethambutol and Pyrazinamide, these cases were labeled as (Multi Drug Resistance) MDR patients. The mean month time for anti-tubercular treatment in these patients was 26.7 ± 17, the maximum being 6 years. All HIV infected patients included in this study were present with pulmonary manifestations.

Overall the detection rate of L-J culture in the applicant's laboratory was 32% (85/265). Similarly, the detection rate of Ziehl-Neelson (Z-N) staining was only 19.6%, and that of Auramine-O (AO) staining 22.3%.(As given in Example 2) However, the sensitivity of AO staining and ZN stained smears on culture proven cases was 75% and 66.6% for MDR cases, 72.7% and 68.2% for new pulmonary tuberculosis cases, 66.6% and 58.3% for tubercular lymphadenitis and 60% and 46.6% in HIV infected pulmonary tuberculosis cases, respectively. Hence the AO stain had a clear edge over Z-N stain and the culture had the highest detection rate.

On culture examination, 86 strains could be isolated from the 85 samples. (For details see example 1& 2) One sample grew two types of colonies one being a rapid grower. All the 86 cultures which were identified as Mycobacterial in nature using standard protocol such as culture examination as exemplified in Example 2.

Genus-specific PCR assay

These cultures were further tested for the presence of Mycobacterium species using molecular tools. The DNA was isolated directly from the above 86 cultures. The DNA isolation was carried out as given in Example 3. All the 86 DNA samples were subjected to genus-specific amplification as given in Example 4. Briefly, DNA from the samples was amplified using 16S rRNA gene primers (genus-specific primers) using suitable buffers. These primers (genus-specific primers) are given below.

Primer 16S rRNA 285: 5' gag agt ttg atc ctg gct cag 3' (SEQ ID NO: 1) and

Primer 16S rRNA 264: 5' tgc aca aca ggc cac aag gga 3. (SEQ ID NO: 2)

After amplification using the above primers the amplified products were separated on 0.9% agarose gel and stained using ethidium bromide. All the samples showed a product of size 1030 bp .Figure 1 shows a representative profile of the PCR assay of some of the samples analaysed All the lanes showed an amplified product of size 1030 bp indicating the presence of *Mycobacterium* species. . This indicated a positive result clearly showing the presence of *mycobacterium* strains as seen in Figure 1. Some of the amplified products were sequenced (29) to confirm whether these sequences are of *Mycobacterial* origin. From the sequence analysis it is clear that all the strains were *Mycobacterium* strains and the details are given in Example 6.

Species-specific PCR assay

All the above 86 DNA samples were further subjected to PCR assay using species-specific primers for the ESAT-6 region. The details of the assay are given in Example 5. Briefly, the 86 DNA samples were amplified using oligonucleotide primer pair having SEQ ID NO: 3 and SEQ ID NO 4. The sequences of the primers are given below:

ESAT-6 F: 5' gcg gat ccc atg aca gag cag cag tgg a 3' (SEQ ID NO: 3) and

ESAT-6 R: 5' ccc aag ctt cct atg cga aca tcc cag tga cg 3' (SEQ ID NO: 4)

The DNA samples along with the primers, DNA polymerse in a suitable buffer were amplified. Out of 86 samples analyzed, (85 plus two types of growth in one patient) only 67 (77.9%) samples showed an amplification product of 320 bp which corresponds to *Mycobacterium tuberculosis* species-specific *esat*-6 gene (See Table 1 for details). Figure 2 shows the amplification profile of some of the samples which were positive for the presence of *Mycobacterium species* based on genus-specific 16s rRNA PCR assay and other conventional method of diagnosis. It is clear from Fig. 2 that all the lanes did not show the presence of amplified product of size 320 bp. Lane 16 did not show any amplification and it corresponds to *M.bovis* strain This clearly indicate that this assay based on *esat* 6 gene is specific for *M.tuberculosis*. Table 2 gives the data of the species specific assay using different strains of *Mycobacterium*. It is clear from Table 2 and Fig. 2 that the PCR assay based on *esat*-6 gene is specific for *M.tuberculosis*.

Sequence analysis of 16Sr RNA genes

The patient, from whom two types of Mycobacterial colonies grew, was on anti-tubercular treatment for the past four years but without any significant clinical benefit. Both the colonies were subjected to colony characterization following the conventional methods

as well as the polymerase chain reaction and 16S rRNA-sequencing. One of the two colonies did not contain the *esat*-6 gene, though genus-specific PCR was positive. The non-tubercular colony, which was ESAT-6 PCR negative, was identified as *M. smegmatis* while the other as *M. tuberculosis* based on sequence analysis of the 16S rRNA genes. The details of the sequencing of the 16S rRNA genes are given in Example 6.The sequence of the *M. smegmatis 16S* rRNA gene (Accession No: AF504932) is different from that of *M. tuberculosis*. The *M.tuberculosis* sequence. obtained by the applicant is same as that in the Gen Bank data base.

Various environmental and human opportunistic Non-tubercular *Mycobacterium* strains were identified by gene sequencing and the sequences have been submitted to GenBank with Accession no AF419854, AF504932, AF504931, AF504926, AF498754, AF498317.

On the basis of genus and species-specific primers used in this study, the Applicants found very high rate of non-tubercular Mycobacteria in HIV infected patients (33.3%, 5/15). It was closely followed by patients with lymphedenitis (29.1%, 7/24) and patients unresponsive to antitubercular treatment (20.8%, 5/24). These findings were highly significant ($p < 0.001$) while comparing the incidence of non-tubercular Mycobacteria in all the above groups with prevalence in new cases (4.5%). All the 18 non-tubercular Mycobacteria have been identified phenotypically and genotypically. The 16S.rRNA gene was amplified from all these strains and sequenced to confirm the absence of M.tuberculosis. in the samples.

The All India Institute of Medical Sciences (AIIMS), New Delhi is a tertiary care hospital and most of the patients are already treated for tuberculosis empirically at local peripheral level. As a result less number of cases visit this hospital directly without taking prior treatment. As our figures show, more than half (52.2%) of these patients were taking treatment but without improvement. The prevalence of non-tubercular Mycobacteria in these patients was significantly ($p < 0.001$) higher than new cases (20.8% vs. 4.5%). This finding is in concurrence with another major study published recently from south India (21). In this study, the prevalence of MDR in newly diagnosed cases of pulmonary tuberculosis was 2.5% while in previously treated cases it was 81.2% of the *M. tuberculosis* isolates (21). Our study also indicates that 79.2% cases were MDR-*M. tuberculosis* while the rest 20.8% cases did not respond because the infection was caused by non-tubercular Mycobacteria.

As expected, in the present study, the prevalence of non-tubercular Mycobacteria was very high in AIDS associated pulmonary tuberculosis. In another study (5) carried out from south India on HIV-TB co-infection, recently showed that in AIDS associated tuberculosis the primary unresponsiveness to anti-tubercular treatment was as high as 33.9%. We also found that our 33.3% patients had non-tubercular Mycobacteria, most of them identified to be *Mycobacterium avium-intracellulare* using 16s rRNA sequencing See Example (6). Since the non-tubercular Mycobacteria are not susceptible to conventional anti-tubercular treatment, it is always desirable to identify these isolates up to the species level, particularly from drug resistant cases. Though, the earlier study from south India (5) did not specify the causative species from unresponsive AIDS patients, our findings are another milestone in this direction, clearly emphasizing the need for speciation of the causative species of Mycobacteria. The present study countermands the general belief that all non-responding cases in India are due to MDR-TB. Our data indicates that more than one quarter of these patients who do not respond to primary ATT are infected with non-tubercular Mycobacteria.

M. tuberculosis has been reported as a leading cause of lymphadenitis in developing world and the USA (26, 33). The data from India is scarce and only a few case reports are available and the speciation is based only on phenotypic features which have several limitations. The high rate (29.2%) of non-tubercular Mycobacteria causing lymphadenitis in the present study could, therefore, be explained on the basis of highly sensitive and *M. tuberculosis* specific molecular method used here. Also most of the non-tubercular Mycobacterium species were identified as *Mycobacterium avium* complex based on 16s rRNA sequencing as given in Example 6. *Mycobacterium* strains were identified by gene sequencing of the 16S rRNA region and the sequences have been submitted to GenBank with Accession no AF419854, AF504932, AF504931, AF504926, AF498754, AF498317

The Mycobacterial DNA detection rate in a multicentric study carried out by these claimants was found to be between 90-96% on all the sputum samples taken from confirmed cases of pulmonary tuberculosis and no false positive were observed as given in Example 7.

The present invention teaches on specific method for detection of *M.tuberculosis*. This invention teaches that the ESAT-6 premiers can be used as rapid and accurate method of identifying the *M.tuberculosis* from the *mycobacterium* isolates obtained either conventional or rapid culture techniques. This will obviate the need of performing several

biochemical methods, which are time consuming, and often not reproducible. The table (1) clearly proves the superiority of esat-6 primers over the biochemical-method of speciation where 4 isolates were incorrectly identified as *M.tuberculosis but* finally identified as non-tubercular mycobacteria. We also propose that these primers can be used to diagnose the tubercular infections, directly on the clinical samples. However, when non-tubercular mycobeterial diseases are expected in higher percentage such as in patients with HIV infection, patients not responding to standard anti-tubercular treatmen' and in Mycobacterial lymphadenitis, these primers should invariably be combined with genus specific primers.

The invention is further exemplified with the following examples which are provided to a person average skill in the art and these examples are not considered to limit the scope of the invention.

EXAMPLES

Example 1

Clinical Samples:

Clinical samples were obtained from patients attending the out patient department (OPD), or hospitalized patients at the All India Institute of Medical Sciences (AIIMS), New Delhi, India. All the samples were processed in the Clinical Microbiology Division, Department of Laboratory Medicine. A total of 3072 patients who attended the general OPD of AIIMS, with complaints of fever, cough and expectoration were investigated to rule out tuberculosis, as a routine work-up. However, only 265 of these patients had clinico-radiological findings suggestive of tubercular etiology and were referred to specialized clinics. These referred patients fulfilled the inclusion criteria as per CDC guidelines and were included in this study. After informed consent of the patients, their routine investigations were carried out and their blood samples were also tested for human Immunodeficiency virus (HIV) infection. The clinical samples investigated for Mycobacterial isolation included sputum, lymph node aspirate or biopsy specimens and pleural fluid, according to the clinical manifestations. In case of sputum, 1-3 samples from each patient were obtained, while for other specimens sampling was done only once. However, repeat samples from the same patient were not counted as different, even if one or all the three samples from one patient were culture positive, it was counted as one only. All samples were processed on the same day of receipt. The sputum samples were decontaminated and concentrated using Petroff's method (12) within 24 hours of receipt.

The decontaminated samples were inoculated on L-J culture slants. One aliquot of the pellet was stored at -20°C until further use. From the remaining pellet slide smears were prepared.

Example 2

Microscopy and routine culture:

The slide smears were air dried and heat fixed. One set of smears was stained with auramine-O (A-O) fluorochrome staining and examined under epifluorescence at 400X using Nikon™ fluorescent microscope and the another set stained with Zeihl-Neelsen staining and examined under oil emersion (1000X). The results and quantification of acid fast bacilli (AFB) were reported as per revised WHO guidelines (33): scanty (1 or 2 AFB in 300 oil fields), 1+ (3-9 AFB in 100 fields), 2+ (1-10 AFB per oil field), or 3+ (10 or more AFB per oil field).

Culture and identifications were made according to the standard method (12, 36) by inoculating on a Lowenstein-Jensen (L-J) slant with 0.2 to 0.5 ml of the decontaminated sputum sample. Cultures were incubated at 37°C till the growth or for 8 weeks whatsoever was early. The cultured Mycobacteria were identified by staining and standard biochemical methods including Niacin, heat stable catalase, aryl sulphatase and Tween-80 hydrolysis tests.

Example 3

Extraction of genomic DNA:

The DNA was isolated directly from the *Mycobacterium* cultures by a procedure which the applicants have been following in the laboratory for the last several years. Briefly: 2-3 loopful (~100mg) of Mycobacterial cultures was transferred to an eppendorf tube containing 200 µl of sterile distilled water. The suspension was incubated at 80°C in a water bath for 20 minutes. To the suspension 200 µl chloroform was added followed by vortexing. The suspension was again incubated at 65 °C for 10 minutes and centrifuged at 9000 rpm for 2 minutes. The clear supernatant containing Mycobacterial DNA was taken for assay.

Example 4

Genus-specific PCR Assay:

Mycobacterial DNA (50ng) was amplified in a 50 µl reaction mixture for the amplification of 16S rRNA gene The reaction mixture contained, 200µM deoxynucleoside triphosphates, dNTPs (Bangalore Genei, Pvt. Ltd., Bangalore, India), 5µl of 10X buffer [100mM TAPS

(pH 8.8), 15mM $MgCl_2$, 500mM KCl and 0.1% gelatin] and 1.5 units of *Taq* DNA polymerase. The working concentration of each primer was 0.5µM. The temperatures used were: 94^0C for 5 minutes; then 25 to 35 cycles of 94^0C for 1 minute, 62^0C for 2 minutes and 72^0C for 2 minutes. A total 40 amplification cycles were carried out followed by final extension at 72^0C for 10 minutes. The PCR products were resolved on a 0.9% agarose gel after ethidium bromide staining. The amplicons of 1030 bp size indicated positive result (Figure 1). Primers for the amplification of a 1030 bp 16S rRNA gene were taken, as described elsewhere (14, 15, 28, and 33). The primers for16S rRNA was:

Primer 16S rRNA 285: 5' gag agt ttg atc ctg gct cag 3' (SEQ ID NO 1) and
Primer 16S rRNA 264: 5' tgc aca aca ggc cac aag gga 3. (SEQ ID NO 2)

Example 5

Species- specific PCR Assay:

All the samples were also subjected to *M. tuberculosis* specific PCR, using primers designed to amplify full length *esat-6* (Rv3875) gene. These novel primers have been designed for the first time for species-specific PCR based diagnosis of *Mycobacterium tuberculosis*. The primers also contained restriction sites for cloning in an expression vector. The primers were:

ESAT-6F: 5' gcg gat ccc atg aca gag cag cag tgg a 3' (Bam HI site underlined) (SEQ ID NO 3) and

ESAT-6R ccc aag ctt cct atg cga aca tcc cag tga cg 3' (Hind III site underlined) (SEQ ID NO 4)

Mycobacterial DNA (50ng) was amplified in a 50 µl reaction mixture for the amplification of the *esat*-6 gene The reaction mixture contained, 200µM deoxynucleoside triphosphates, dNTPs (Bangalore Genei, Pvt. Ltd., Bangalore, India), 5µl of 10X buffer [100mM TAPS (pH 8.8), 15mM $MgCl_2$, 500mM KCl and 0.1% gelatin] and 1.5 units of *Taq* DNA polymerase. The working concentration of each primer was 0.5µM. The temperature cycles used were: 30 cycles of 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C; followed by final extension at 72°C for 10 min The PCR products were resolved on a 0.9% agarose gel after ethidium bromide staining. The products were electrophoreses in 1.5% agarose gels, to resolve the PCR product of 320bp (Figure 2) Further the PCR amplified fragment obtained using primers having SEQ ID NO 3 and SEQ ID NO.4 from

genomic DNA of a local clinical isolate of *M.tuberculosis* was cloned in a plasmid vector and sequenced. The nucleotide sequence obtained was identical to that published by Cole *et al* (4). The sequence has been deposited with the GenBank with accession no. AF420491.

Example 6: Sequencing of 16S r RNA genes of *Mycobacteria* species

The PCR fragment obtained using primers (SEQ.ID.NO.1 AND 2) from genomic DNA of a local clinical isolate of *M. tuberculosis* was cloned into an intermediate vector pGEM-T easy and its nucleotide sequence ascertained. Several of the clinical samples which showed positive amplification after the species-specific amplification were analyzed using the sequencing of the 16S rRNA genes to confirm the presence of *Mycobacteria tuberculosis*. The clinical samples which did not show amplification using primers based on *esat*-6 gene were analysed using the 16S rRNA gene sequences. It was clear that the samples which did not show amplification with *esat*-6 gene were not Mycobacteria *tuberculosis*. Most of them were identified to be *Mycobacterium avium-intracellulare* based on 16S. rRNA sequences. The various Non-tubercular Mycobacteria were identified by gene sequencing and the sequences have been submitted to GenBank with Accession No.AF419854, AF504932, AF504931, AF504926, AF498754, and AF498317. Since the Non-tubercular mycobacteria are not susceptible to conventional anti-tubercular treatment, it is always desirable to identify these isolates upto species level, particularly from drug resistant cases.

Example 7: Detection of Mycobacterium *tuberculosis from confirmed clinical samples*

From the clinical study undertaken, 50 clinical samples were used for detection of Mycobacterium. All the above 50 sputum samples taken were from confirmed cases of pulmonary tuberculosis. The sputum samples were subjected to two PCR amplifications as given in Example 4 and 5 The PCR amplified Products were separated on agarose gel as given in example 4 and 5. .The amplified products were detected by staining with ethidium bromide. Most of the 50 samples showed amplification of a DNA product of 320bp in length. The data from the clinical samples is shown in Table 3

Example 8: Detection of different species of *Mycobacteria*

The PCR assay was also conducted on different species of *Mycobacteria*. The different Mycobacterial strains used in this study are shown in Table 2. DNA samples from the different strains were isolated from cultures as given in Example 3. The various DNA samples were subjected to both genus-specific and species–specific PCR assay. The genus –

specific assay was carried out using 16S rRNA primers having SEQ ID NO: 1 and 2 as given in Example 4. The species-specific assay was carried out using species-specific primers having SEQ ID NO: 3 and SEQ ID NO: 4 as given in Example 5. The PCR amplified products were separated on agarose gels and analyzed. The data from these experiments is shown in Table 2.

Table 2 clearly shows that the *esat-6* species-specific primers were highly specific for *M. tuberculosis*. Even though primers specific for *Mycobacterium tuberculosis* complex (which includes *M. bovis*) have been reported, ours is the first invention which can differentiate *Mycobacterium tuberculosis from M .bovis*. It is important to differentiate *Mycobacterium tuberculosis from M .bovis* as *M .bovis* can be prevalent due to post BCG vaccination infection. In India, BCG (Bacillus of Calmette and Guerin) vaccine is administered in early childhood to all children. The vaccine contains mutated BCG strain of *M. bovis*. So far there are no rapid and direct molecular methods of making exclusive diagnosis of *M. tuberculosis* infection minus *M. bovis*. Moreover, the limitation of antibody detection assays using ESAT-6 antigen is enormous in India as stated earlier. The *Mycobacterium bovis* is also an important cause of tuberculosis in animals (cattle) of India and traditionally the Indian farmers who consume raw cow milk will have antibodies against these bacteria. Therefore the antibody detection methods will give false positive report of tuberculosis in otherwise healthy individuals and many patients may undergo unnecessary anti-tuberculosis treatment while not treating the actual disease. Therefore, our invention will revolutionize the *M. tuberculosis* species-specific diagnosis by using multiplex PCR (using 16S rRNA and *esat-6* primers) directly on the clinical samples or using single *esat-6* primers on the culture isolates. 1.

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Table 1. Detection rate of Mycobacterial etiology in 4 patient groups using various conventional and molecular methods

Patient Group	Culture isolates	Biochemical identification		PCR identification			
				16S rRNA		*Esat*-6	
		M.tb	NTM	+	-	+	-
AIDS	15	10 (66.6%)	5 (33.3%)	15	0	8	7
Lymphadenitis	24	19 (79.2%)	5 (20.8%)	24	0	17	7
MDR-TB	24+1*	19 (76.0%)	6 (24.0%)	25	0	19	6
Rx Naïve	22	21 (95.5%)	1 (4.5%)	22	0	21	1
Total	85+1*	69 (81.2%)	16 (18.8%)	85	0	65	20

*from one patient, two types of isolates were obtained. One was identified as *M. tuberculosis* and the other as *M. smegmatis*.

Table 2

Mycobacterial species	16S rRNA PCR result	6 PCR result
M. tuberculosis	+	+
M. bovis (BCG)	+	-
M. avium	+	-
M. smegmatis	+	-
M. duvalli	+	-
M. fortuitum	+	-
M. celatum	+	-
M. austroafricanum	+	-
M. flavescence	+	-

Table 3: The relative sensitivity of various diagnostic methods

N=50	Smear	Culture	16srRNA	ESAT-6
Positive	18	31	48	45/48
Negative	32	19	10	3/48*

*None of the 16SrRNA PCR negative sample was found ESAT-6 PCR positive

I / We Claims

1. An oligonucleotide primer pair having SEQ ID NO: 3 and SEQ ID NO: 4 for amplification of Early Secretory Antigenic Target (*esat*)-6-gene of *Mycobacterium* species.

1. A method for detecting *M .tuberculosis* in a sample based on the amplification of *esat*-6 gene, the said method comprising the steps of :
   a) isolating DNA template from the sample,
   d) amplifying the DNA template by adding a reaction buffer, oligonucleotide primer pair having SEQ ID NO: 3 and SEQ ID NO: 4, and heat stable DNA polymerase to obtain an amplified DNA product, and
   e) subjecting the amplified DNA product of step (b) to separation, and staining to detect the presence of amplified DNA product wherein the presence of amplified DNA product is indicative of *Mycobacterium tuberculosis* in the sample.

3. A method according to claim 2, wherein the sample is either clinical sample or culture sample.

4. A method according to claim 3, wherein the clinical samples is selected from a group of sputum, bronchoalveolar, lavage fluid, pleural fluid, ascetic/peritoneal fluid, cerebrospinal fluid (CSF), pus fecal matter, urine, amniotic fluid, menstrual blood, peripheral blood or other body fluids, lymph node, pus or other aspirate, and tissue biopsies.

5. A method as claimed in 2 wherein in step (b) the amplification is by polymerase chain reaction.

6. A method as claimed in 2 wherein the amplification consists of 25-35 cycles of amplification.

7. A method according to claim 2, wherein in step (b) the heat stable DNA polymerase is *Taq polymerase*.

8. A method as claimed in 2 wherein in step (c) the separation is done preferably by gel electrophoresis.

9. A method as claimed in 2 wherein in step (c) the staining is by ethidium bromide.

10. A method as claimed in 2 wherein in step (c) the amplified DNA product is 320 base pair in length.

11. A diagnostic kit for detection of *Mycobacterium tuberculosis*, from other species of *Mycobacteria* comprising of oligonucleotides primers having SEQ ID NO: 3 and SEQ ID NO: 4, all four deoxyridonucleotide triphosphate (dNTPs), reaction buffer, *Taq polymerase*, DNA marker, positive and negative control and instruction manual.

12. A method for detecting *M. tuberculosis* based on amplification wherein, the said method comprising the steps of:

i. amplifying the 16s rRNA region from the isolated DNA template using the primer pair having SEQ ID NO: 1 and SEQ ID NO: 2 to obtain first amplified product using conventional method,

ii. detecting the amplified product of step (a) wherein the presence of 1030 base pair amplified DNA product is indicative of positive sample for the presence of *Mycobacterium* species,

iii. employing the DNA from the positive samples identified from step (b) for further detection of *M. tuberculosis* based on the amplification of *esat*-6 gene,

iv. amplifying the *esat*-6 gene using the primer pair having SEQ ID NO: 3 and SEQ ID NO: 4 to obtain second amplified product using method as claimed in claim 2, and

v. detecting the amplified product of step (d) wherein the presence of 320 base pair is indicative of *Mycobacterium tuberculosis* in the sample and absence is indicative of other *Mycobacterium* species.

13. A method according to claim 12, wherein the DNA template is obtained either from clinical sample or from culture sample.

14. A method according to claim 13, wherein the clinical sample is selected from a group of sputum, bronchoalveolar, lavage fluid, pleural fluid, ascetic/peritoneal fluid, cerebrospinal fluid (CSF), pus fecal matter, urine, amniotic fluid, menstrual blood, peripheral blood or other body fluids, lymph node, pus or other aspirate, and tissue biopsies.

15. A method as claimed in 12 wherein the amplification is by polymerase chain reaction.

16. A method according to claim 15 wherein the amplification is by heat stable DNA polymerase such as *Taq polymerase*.

17. A method as claimed in 12 wherein in step (i) the amplification consists of 30-40 cycles of amplification.

18. A method as claimed in 12 wherein in step (iii) the amplification consists of 25-35 cycles of amplification

19. A method for detecting *M..tuberculosis* based on the amplification of *esat*-6 gene, substantially as herein described with reference to examples and figures.

DATED THIS 22ND DAY OF DECEMBER 2004

T.SRINIVASAN
AGENT FOR THE APPLICANT

TO
THE CONTROLLER OF PATENTS,
PATENT OFFICE BRANCH, AT NEW DELHI

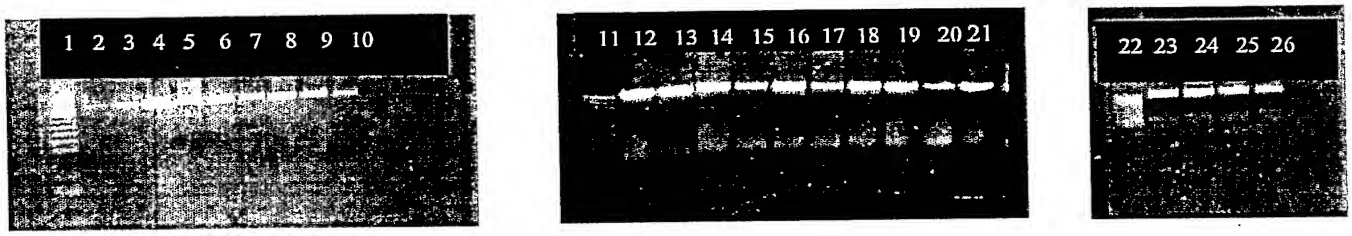
Applicant Name: **All India Institute Of medical sciences**

Application No:

22 DEC 2004

Sheet 1/2

Figure-1



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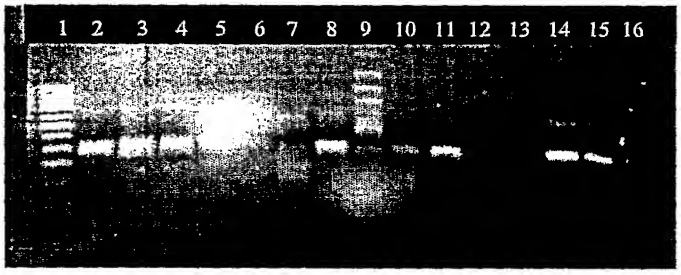
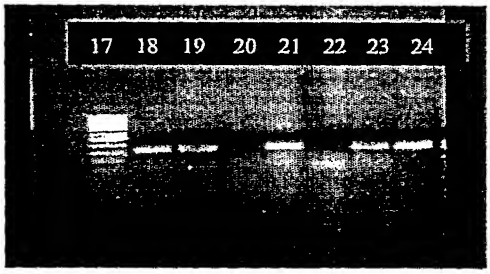
Agent for the Applicants

Applicant Name: **All India Institute Of medical sciences**

Application No:

Figure-2





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